Control of Cell Adhesion and Growth with Micropatterned Supported Lipid Membranes

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In this work, phospholipid bilayers are employed as biomimetic coating materials to modulate the adhesion and growth of cells on solid substrates. A variety of lipid compositions and charge densities are examined. Culturing cells on these supported membranes reveals that fluid lipid bilayers generally block cell adhesion with a notable exception provided by membranes containing phosphatidylserine, which strongly promote adhesion and growth. This dichotomy is utilized with micropatterned membrane technology to selectively direct cell growth to specified regions on a substrate. Lipid composition in micropatterned membrane arrays is demonstrated to be a simple and effective means of patterning cell growth on surfaces.

Introduction

There is intensifying interest in the use of micropatterned biofunctional materials to organize and control the growth of cells on surfaces. Such materials offer attractive capabilities for cell-based biosensor technology, microfluidic cell screening systems, tissue engineering applications, and fundamental research.

A number of studies have demonstrated that patterning chemical and physical characteristics of a surface can significantly influence cellular behavior.[1−7] For example, self-assembled monolayers (SAMs) of alkanethiols on gold that present poly(ethylene glycol) (PEG) moieties effectively resist protein adsorption, thus blocking extracellular matrix (ECM) deposition and subsequent cell adhesion. In contrast, hydrophobic SAMs readily adsorb proteins, including those of the ECM, and consequently promote cell adhesion.[3,8−11] Alternatively, an effective surrogate ECM can be provided by chemically coupling an adhesion-promoting peptide, such as arginine−glycine−aspartate (RGD), to the surface.[12−14] Surfaces patterned with adhesive and resistant SAMs (by microcontact printing[3,6,11,10] or photolithography[15]) have been successfully used to guide and control the deposition, growth, and death of cells.

Supported phospholipid membranes present a naturally biofunctional surface which can also modulate cell behavior. Continuous single bilayer membranes can be assembled on solid substrates, such as silica, by spontaneous fusion of lipid bilayer vesicles or by subsequent deposition of two Langmuir monolayers.[16−18] A thin (∼10 Å) film of water lubricates the membrane−solid interface allowing free lateral diffusion within the fluid lipid bilayer (Figure 1).[19−21] Supported membranes displaying specific proteins have proven to be effective artificial cell surfaces in a number of immunological studies involving interactions with living cells (T-lymphocytes,[22−25] neutrophils[26,27]). In addition to these remarkable capabilities, supported membranes are readily patterned by a variety of microfabrication techniques including substrate-imposed micropartitioning,[28−30] electric field induced reorganization,[31−34] and microcontact stamping.[35]

Here, we explore the use of lipid composition in supported membranes as a means of controlling the adhesion and growth of cells on solid substrates. Two naturally adherent cell lines, HeLa (human cervical carcinoma) and NIH3T3 (mouse fibroblast), were cultured on a panel of supported membranes covering a range of lipid compositions and charge densities. All of the mem-

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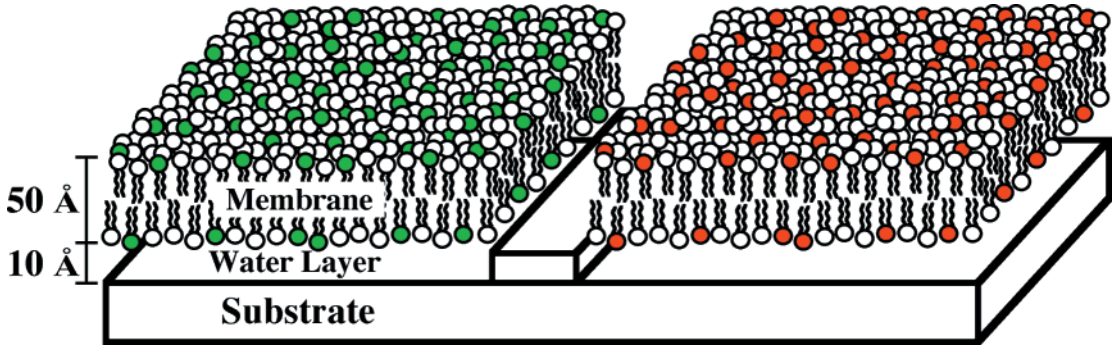

Figure 1. Schematic diagram of a supported membrane. A ~10 Å water layer separates the lipid bilayer from the substrate allowing free lateral diffusion of lipids within the membrane. Patterns of materials, such as chrome, on the silica substrate act as barriers to lateral diffusion, enabling arrays of different membrane types to be displayed side-by-side.

brane compositions examined block cell adhesion except those containing phosphatidylserine (PS). PS is known to promote the pathological adhesion of erythrocytes (abnormally expressing PS in the outer leaflet of their membrane) to endothelium in conditions such as sickle cell disease, falciparum malaria, and diabetes.[36−38] In our studies, PS-containing membranes strongly promoted adhesion and growth in both cell lines studied. This finding allowed us to use lipid bilayer patterning technology to selectively direct cell adhesion to specified elements in a membrane microarray.

Results and Discussion

The ability of supported membranes to modulate the adhesion of cells to a solid substrate was examined by culturing cells on supported membrane coated silica. Membranes were formed on silica coverslips or micropatterned chips under sterile conditions in cell culture plates. The membrane spreading buffer was exchanged for culture media as detailed in the methods section, and ~50 000 cells/cm^2 were inoculated in each well. Observations were made after 6 and 24 h as well as longer time periods in some experiments.

Cell adhesion was characterized on egg-phosphatidylcholine (egg-PC) membranes doped with a variety of negatively and positively charged lipids. Doping ratios of 5 mol % were studied for each of the charged lipids; positively charged DAP and negatively charged PS were also examined at 3 and 9 mol % doping ratios. In all cases, PS-containing membranes promoted cell adhesion while other compositions effectively blocked cell adhesion. The results from a typical adhesion assay are illustrated in Figure 2. Initial adhesive contacts between cells and the substrate formed within 6 h when the supported membranes contained PS. At this stage, the cells were evenly distributed over the substrate and remained fixed in place under mild shaking (Figure 2A). After 24 h, focal adhesion sites were well formed and cells exhibited morphology consistent with that observed in tissue culture plates (Figure 2B). In contrast, membranes lacking PS blocked cell adhesion. After 6 h, cells tended to clump together and were not fixed to the substrate as determined by their movement under mild shaking. Little or no change in this condition was observed after 24 h (Figure 2C,D). In all experiments, cells that settled on the plastic tissue culture plate grew normally, thus providing an internal positive control for cell viability. Results for the specific lipids and cells studied are listed in Table 1.

Lateral fluidity of supported membranes is one of their most distinctive features and can serve as a stringent test of membrane integrity. Long-range mobility of membrane components is easily observed by fluorescence recovery after photobleaching (FRAP). Simple FRAP experiments were performed on supported membranes during cell culture to affirm that the membranes maintain their continuity and fluidity in the culture environment. Qualitative FRAP observations were made by illuminating a 100 μm octagonal section of the membrane with full power fluorescence excitation from the microscope light source. An outline of the fluorescence photobleach zone is drawn on a brightfield phase contrast image of the surrounding area in Figure 3A. A simultaneous phase contrast and fluorescence image taken during the photobleach period is shown in Figure 3B (3× digital magnification relative to Figure 3A). Emission from unbleached probe lipids within the octagonal photobleach zone is visible in addition to outlines of the cells from low-intensity phase contrast illumination over the entire field of view. The halo of fluorescence visible just inside the photobleach zone is from unbleached probe lipids diffusing into this brightly illuminated region during the exposure.

A significant fraction of the fluorescently tagged probe lipids within the illumination spot were photobleached within a few minutes. The ensuing fluorescence recovery by diffusive mixing was monitored by imaging the fluorescence pattern over a wider area. Figure 3C contains a series of three fluorescence images, covering the full field of view shown in Figure 3A. These images were taken with brief exposures 1, 10, and 30 min after the bleach, as labeled. The distribution of cells seen in Figure 3A remained constant throughout this time period. Diffusive spread of the photobleached spot is clearly visible and the time scale is in rough agreement with the typical diffusion coefficient (~4 μm^2/s) of lipids in supported membranes.[31] These observations confirm that supported membranes remain intact under standard cell culture conditions

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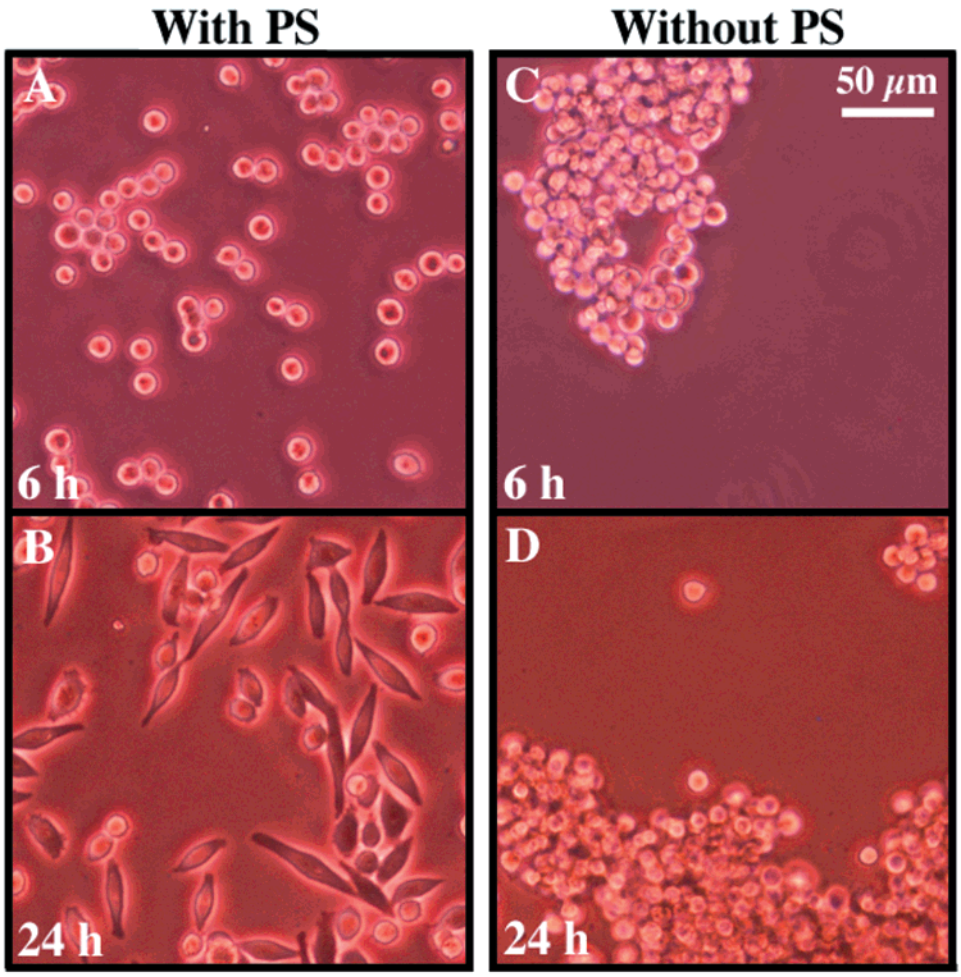

Figure 2. HeLa cells cultured on supported membranes. Membranes in (A) and (B) are 5% PS/94% PC/1% NBD-PE; those in (C) and (D) are 5% DAP/94% PC/1% NBD-PE. Observations were made at 6 and 24 h as labeled.

Table 1. Cell Adhesion Data[a]

lipid	charge	adhesion	cell lines tested
PS	−	yes	HeLa, NIH3T3
PA	−	no	HeLa
PG	−	no	HeLa
PI	−	no	HeLa
DAP	+	no	HeLa, NIH3T3
TAP	+	no	HeLa
DDAB	+	no	HeLa
ethyl-PC	+	no	HeLa

[a] Cell adhesion data were collected on egg-PC supported membranes containing 5 mol % of the charged lipid under study. See the experimental protocol section below for definitions of abbreviations.

(DMEM + 10% FCS) for at least 24 h; membranes were found to last much longer in some cases. Additionally, it is seen that cells resting on the supported membrane do not significantly influence the fluidity or continuity of the underlying lipid bilayer.

There is evidence that the focal adhesion sites observed in cells growing on the supported membrane effectively penetrate the fluid lipid bilayer and anchor on the solid substrate. This is supported by the stretched morphology of the cells, which indicates a tensile force between the anchored focal adhesion sites[39] (see Figures 2B and 4B). The supported membrane is fluid and thus intrinsically incapable of withstanding any such forces. Lateral forces applied to fluid membranes induce motion of the lipids and readily produce large-scale (>100 μm) rearrangements of the membrane.[31−33] Presumably, this anchoring of the cells results from ECM deposition by the cellular process sometimes referred to as substrate remodeling. PS in the supported membrane likely provides the initial link that enables the cell to subsequently form a firm adhesive contact with the underlying solid substrate. In this way, PS modulates the interaction between cells and supported membrane coated substrates in a chemically specific manner.

In the second phase of this investigation, PS-mediated cell adhesion was used to pattern cell growth on supported membrane surfaces. Membrane microarrays displaying alternating corrals of PS-containing and PS-free membrane were deposited on prefabricated substrates with either 200 or 500 μm grid sizes. The membrane within each corral in the microarray is fluid, while grids of chrome barriers on the silica substrate prevent mixing between separate corrals. Different fluorescently labeled lipids were incorporated in the various membrane types, allowing them to be distinguished in the microarray.

Cells cultured on microarray surfaces selectively adhered to and grew on the PS-containing membrane corrals. Figure 4 illustrates fluorescence (A) and phase contrast (B) images of a four-corral section of a microarray. The red fluorescence from the membrane in the upper two corrals identifies them as PS-containing (5% PS/94% PC/1% Texas Red-PE), while the membrane in the lower two

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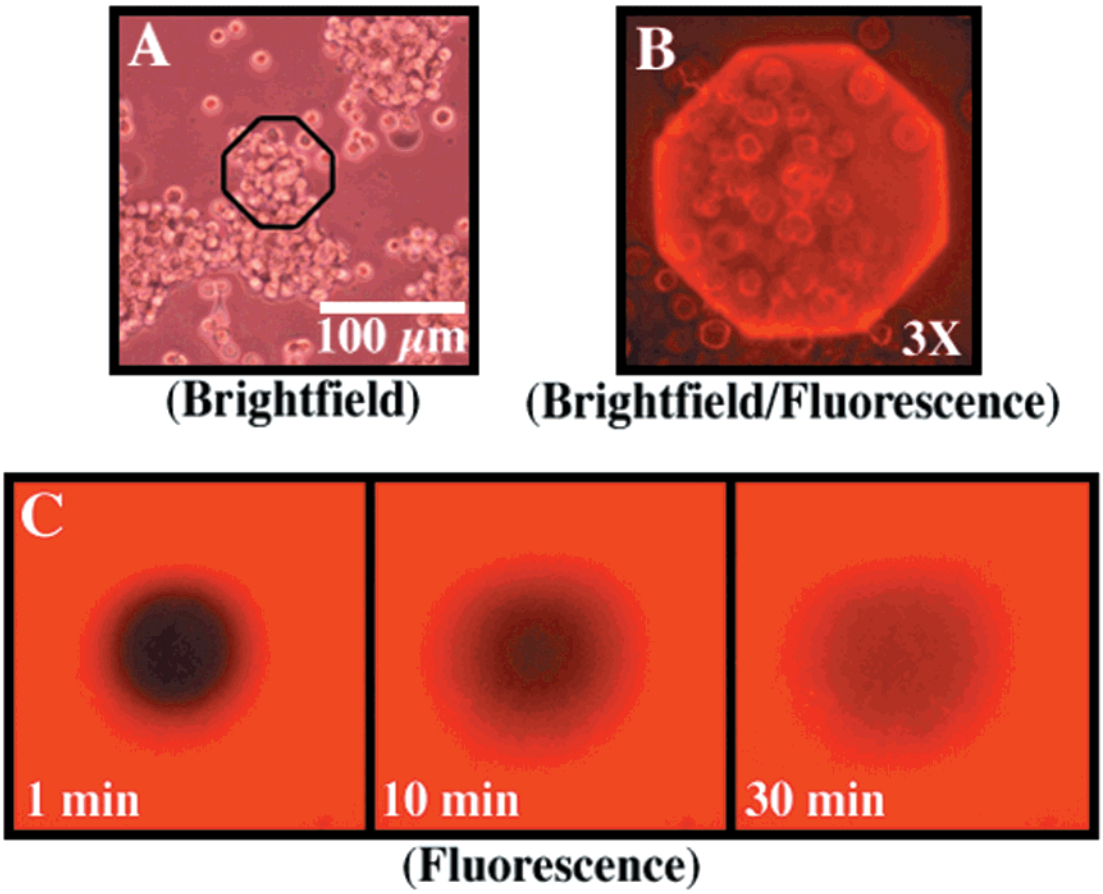

Figure 3. Outline of a FRAP experiment to confirm membrane fluidity and integrity. (A) Brightfield phase contrast image of cells indicating position of bleach spot. (B) The fluorescence excitation is projected in an octagonal spot during the bleach period. A simultaneous phase contrast and fluorescence image is shown at 3× (digital) magnification relative to the scale in (A) and (C). (C) Series of three fluorescence images illustrating uniform diffusive recovery of the photobleached spot. These membranes were fluorescently labeled with Texas Red-PE (1%).

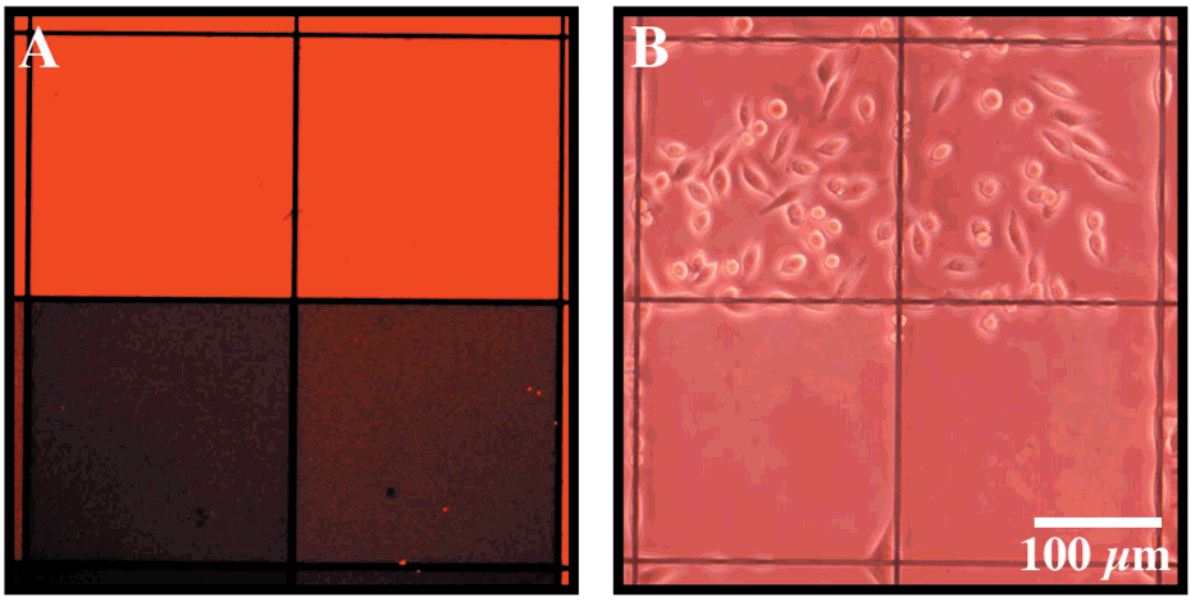

Figure 4. Patterned cell growth on membrane microarrays. (A) Fluorescence image of four membrane corrals in an array. The membrane in the upper two corrals is 5% PS/94% PC/1% Texas Red-PE, while the lower two corrals contain 5% PG/94% PC/1% NBD-PE membranes. Some membrane mixing occurs during the deposition process. (B) Phase contrast image of the same four corrals in (A) illustrating cell positioning. (A) and (B) were both taken 24 h after inoculation.

corrals is PS-free (5% PG/94% PC/1% NBD-PE). The corresponding phase contrast image illustrates the distinct segregation of the cells onto the PS-containing membrane corrals. The nearly complete lack of cell deposition on the identically charged PG-containing membrane (lower two corrals in Figure 4) underscores the chemical specificity of the PS effect. In multiple experiments with various membrane combinations, cells were observed to proliferate

to near confluence on the PS-containing membrane while PS-free membrane corrals remain essentially devoid of cells.

The use of PS as the adhesion-promoting molecule in fluid membranes creates a number of attractive micropatterning opportunities. For example, the vesicle fusion process can be used to deposit supported membranes in enclosed spaces such as the interior capillary walls of a microfluidic chip. Furthermore, laterally applied electric fields can generate spatial patterns, such as continuous concentration gradients or localized enrichments of the negatively charged PS, which can be reorganized dynamically.[31−34] PS-mediated control of cells with supported membranes is simple and provides a variety of unique capabilities, which complement existing cell patterning technologies.

Experimental Protocol

Planar supported bilayers were formed by fusion of small unilamellar vesicles (SUV) with clean silica substrates.[40] A lipid solution in chloroform was evaporated onto the walls of a round-bottom flask, which was then evacuated overnight. Lipids were resuspended in distilled water by vortexing moderately for several minutes. The lipid concentration at this point was around 3 mg/mL. The lipid dispersion was then probe sonicated to clarity on ice, yielding SUV. The SUVs were purified from other lipid structures by ultracentrifugation for 2 h at 192 000*g*. SUVs were stored at 4 °C and typically were stable for a few weeks to several months. l-α-Phosphatidylcholine from egg (egg-PC), phosphatidylserine from brain (PS), dipalmitoylphosphatidic acid (PA), distearoylphosphatidylglycerol (PG), phosphatidylinositol from soybean (PI), 1,2-dioleoyl-3-dimethylammonium-propane (DAP), 1,2-dioleoyl-3-trimethylammonium-propane (TAP), dimethyldioctadecylammonium bromide (DDAB), 1,2-dioleoyl-*sn*-glycero-3-ethylphosphocholine (ethyl-PC), and *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, ammonium salt (NBD-PE), were obtained from Avanti Polar Lipids (Alabaster, AL). *N*-(Texas Red sulfonyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt (Texas Red DHPE), was obtained from Molecular Probes (Eugene, OR).

Micropatterned substrates were fabricated on borofloat glass wafers (diameter = 100 mm; thickness = 0.7 mm) from Precesion Glass & Optics (Santa Ana, CA). A 1000 Å layer of chrome was deposited on the wafers by vapor deposition. The wafers were then spin coated with Shipley positive photoresist and exposed through a photomask (Photosciences, Torrence, CA) by contact lithography. The resist was developed, and the chrome was etched back with CR-4 chrome etch from Cyantek Corp. (Fremont, CA). The wafers were then cut into 9 mm square chips which are reusable.

Silica coverslips or micropatterned chips were cleaned by soaking for 20 min in freshly prepared piranha solution (3:1 mixture of sulfuric acid and 30% hydrogen peroxide). The substrates were then rinsed under DI water and blown dry with compressed air. Prior to supported membrane deposition, spreading solutions were prepared by mixing the SUV suspensions in equal ratios with PBS. For deposition onto coverslips, a 30 μL drop of membrane spreading solution was placed in the center of a well in the cell culture plate and a substrate was immediately laid on top of the drop. Within one minute, the well was filled with PBS. The substrate was then carefully flipped over to expose the membrane surface (membranes must be kept submerged at all times). Each well was flushed several times with PBS to remove excess vesicles and then flushed with cell culture media. Array deposition on micropatterned substrates was carried out by direct pipetting.[41]

NIH3T3 and HeLa cells were cultured in Dulbecco's modified Eagles medium (DMEM) containing 10% fetal calf serum (FCS). Cells were grown in a 37 °C incubator with 5% CO_2 atmosphere. Cells were washed, trypsinized, and resuspended in DMEM with 10% FCS. An average of 200 000 cells were added per sample (∼50 000 cells/cm^2).

Imaging of the membranes, membrane microarrays, and cells was performed on a Nikon Diaphot 200 inverted microscope using a 10× Phase 1 DL objective. The brightfield images of the cells were obtained utilizing the phase contrast mode. Fluorescence images were taken with the same objective in fluorescence mode using a 100 W mercury arc lamp. The supported membranes were fluorescently labeled with 1 mol % of either Texas Red- or NBD-tagged lipids. Images were recorded with a digital color CCD camera (Diagnostic Instruments model 1.3.0) which was driven by the manufacturer's Spot image collection software.

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